Labeled Ligands for Lectin-Like Oxidized Low-Density Lipoprotein Receptor (LOX-1)

BACKGROUND OF THE INVENTION

1. Field of the Invention

[0001] Embodiments of the present invention relate in general to compounds labeled with imaging agents that also are capable of binding lectin-like oxidized low-density lipoprotein (LOX-1). The LOX-1 receptor compounds are molecules that bind to the LOX-1 receptor, which is over-expressed in atherosclerotic lesions and rheumatoid arthritis. The detectable labels include any detectable label, preferably radionuclides for nuclear scintigraphy or positron emission tomography (PET), paramagnetic metal ions or superparamagnetic particles for magnetic resonance imaging (MRI), heavy metal ions for X-ray or computed tomography (CT), gas-filled microbubbles for targeted ultrasonography (US), or optical dyes for optical imaging, porphyrins or texaphyrins for NMR, fluoresent imaging or photodynamic therapy. The labeled compounds are useful for the diagnosis and monitoring of inflammation and diseases in which inflammation plays a role such as various cardiovascular diseases including but not limited to atherosclerosis, vulnerable plaque and coronary artery disease as well as rheumatoid arthritis.

2. Description of Related Art

[0002] Cardiovascular diseases are the leading cause of death in the United States, accounting annually for more than one million deaths. Atherosclerosis is the major contributor to coronary heart disease and is a primary cause of non-accidental death in Western countries (Coopers, E. S. Circulation 1993, 24, 629-632; WHO-MONICA Project. Circulation 1994, 90, 583-612). Considerable effort has been made in defining the etiology and potential treatment of atherosclerosis and its consequences, including myocardial infarction, angina, organ failure and stroke. Despite these efforts, there are many unanswered questions including how and when atherosclerotic lesions become vulnerable and life-threatening, the best point of intervention, and how to detect and monitor the progression of lesions.

[0003] It is well-documented that multiple risk factors contribute to atherosclerosis. Such risk factors include, for example, hypertension, elevated total serum cholesterol, high levels of low density lipoprotein (LDL) cholesterol, low levels of high density lipoprotein (HDL) cholesterol, diabetes mellitus, severe obesity, and cigarette smoking (Orford *et al.*, *Am. J. Cardiol.* 2000, 86 (*suppl.*) 6H-11H). To date, treatment of atherosclerosis has been focused on lowering cholesterol levels and modifying lipids. However, recent studies have indicated that 40% of deaths due to coronary disease occurred in men with total cholesterol levels of below 220 mg/dl. (Orford *et al*).

[0004] In atherogenesis, elevated plasma levels of LDL lead to the chronic presence of LDL in the arterial wall. The modified LDL activates endothelial cells, which attract circulating monocytes (Orford *et al.*). These monocytes enter the vessel wall, differentiate into macrophages, and subject the modified lipoproteins to endocytosis through scavenger receptor pathways. This unrestricted uptake eventually leads to the formation of lipid-filled foam cells, the initial step in atherosclerosis. If the macrophage is present in an environment that is continually generating modified LDL, it will accumulate lipid droplets of cholesteryl esters, continuing until the macrophage dies from its toxic lipid burden. The released lipid then forms the acellular necrotic core of the atherosclerotic lesion. Subsequent recruitment of fibroblasts, vascular smooth muscle cells, circulating monocytes, and T-lymphocytes complete the inflammatory response and the formation of the mature atherosclerotic plaque. Macrophage-derived foam cells are concentrated in the shoulders of plaques, where their secreted proteinases and collagenases may contribute to plaque rapture, which may lead to a fatal thrombotic event.

[0005] The progression of coronary atherosclerotic disease can be divided into five phases (Fuster *et al. N. Engl. J. Med. 1992, 326,* 242-250). Phase 1 is represented by a small plaque that is present in most people under the age of 30 years regardless of their country of origin. Phase 1 usually progresses slowly (types I to III lesions). Phase 2 is represented by a plaque, not necessarily very stenotic, with a high lipid content that is prone to rupture (types IV and Va lesions). The plaque of phase 2 may rupture with a predisposition to change its geometry and to the formation of mural thrombus.

These processes, by definition, represent phase 3 (type I lesion), with a subsequent increase in stenosis, possibly resulting in angina or ischemic sudden death. Phase 2 plaque is not adequately stenotic to create symptoms or even be detected by commonly employed diagnostic techniques such as a cardiac angiogram. It is estimated that 60% of all deaths due to heart attacks result from rupture of a non-stenotic plaque. Accordingly, a diagnostic protocol to detect such plaques would have great value for detecting at risk but asymptomatic patients.

[0006] The ability to detect, quantitate, and monitor atherosclerotic plaque formation is of major clinical importance due to the progression of these plaques to stable coronary artery disease, or to the occurrence of acute ischemic syndromes caused by the rupture of vulnerable plaque. Imaging modalities for the detection of atherosclerotic lesion and thrombosis associated with plaque rupture exist and are described, for example, in Vallabhajosula, S. and Fuster, V., *J. Nucl. Med. 1997,* 38, 1788-1796; Marmion, M. and Deutsch, E., *J. Nucl. Biol. Med. 1996,* 40, 121-131; Cerqueira, M. D., *Seminars Nucl. Med. 1999,* 29, 339-351; Narula, J., *J. Nucl. Cardiol. 1999,* 6, 81-90; Narula, *J. Nucl. Med. Commun. 2000,* 21, 601-608; Meaney *et al. J. Magn. Reson. Imaging 1999,* 10, 326-338; Knopp *et al. J. Magn. Reson. Imaging 1999,* 10, 314-316; Goyen *et al. Eur. J. Radiol. 2000,* 34, 247-256; Becker *et al. Eur. Radiol. 2000,* 10, 629-635).

[0007] Several invasive and noninvasive techniques are routinely used to image atherosclerosis and to assess the progression and stabilization of the disease. These include coronary angiography, intravascular ultrasound angioscopy, intravascular magnetic resonance imaging, and thermal imaging of plaque using infrared catheters. These techniques have been used successfully to identify vulnerable plaques. However, these techniques are generally invasive, requiring surgery, insertion of probes, cameras, or other invasive procedures.

[0008] Soluble markers, such as P-selectin, von Willebrand factor, Angiotensin-converting enzyme (C146), C-reactive protein, D-dimer (Ikeda *et al., Am. J. Cardiol., 1990,* 65, 1693-1696), and activated circulating inflammatory cells are found in patients with unstable angina pectoris, but it is not yet known whether these substances predict

infarction or death (Mazzone *et al.*, *Circulation, 1993,* 88, 358-363.). It is known, however, that the presence of these substances cannot be used to locate the involved lesion.

[0009] Temperature sensing elements contained in catheters have been used for localizing plaque on the theory that inflammatory processes and cell proliferation are exothermic processes. For example, U.S. Patent No. 4,986,671 discloses a fiber optical probe with a single sensor formed by an elastic lens coated with light reflective and temperature dependent material over which is coated a layer of material that is absorptive of infrared radiation. Such devices are used to determine characteristics of heat or heat transfer within a blood vessel. The devices measure parameters including the pressure, flow, and temperature of the blood in a blood vessel. U.S. Patent No. 4,752,141 discloses a fiberoptic device for sensing temperature of the arterial wall upon contact. However, discrimination of temperature by contact requires knowing where the catheter is to be placed. These techniques using catheters or devices are invasive, and sometimes may result in or trigger plaque formation or rupture.

[0010] An angiogram simply reflects luminal diameter and provides a measure of stenosis with excellent resolution. An angiogram, however, does not image the vessel wall or the various histopathological components. Nevertheless, this technique has become the mainstay of the diagnosis of coronary, carotid, and peripheral artery lesions (Galis *et al*, *Proc. Acad. Sci. USA, 1995,* 92, 402-406; Ambrose, J. A. In: Fuster, V. (Ed.); *Syndromes of Atherosclerosis: correlations of clinical imaging and pathology*; Armonk, NY: Futura Publishing Company, Inc., 1996, 105-122; Kohler, T. R. In: Fuster, V. (Ed.); *Syndromes of Atherosclerosis: correlations of clinical imaging and pathology*; Armonk, NY: Futura Publishing Company, Inc., 1996, 205-223; Dinsmore, R. E. and Rivitz, S. M. In: Fuster, V. (Ed.), *Syndromes of Atherosclerosis: correlations of clinical imaging and patholo*gy. Armonk, NY: Futura Publishing Company, Inc., 1996, 277-289), and is the "gold standard" for anatomic diagnosis despite limited specificity and sensitivity.

[0011] An angiogram may be useful for predicting a vulnerable plaque, since low-shear regions opposite flow dividers are more likely to develop atherosclerosis (Ku *et al.*,

Atherosclerosis 1985, 5, 292-302). However, most patients who develop acute myocardial infarction or sudden death have not had prior symptoms, much less an angiography (Farb *et al., Circulation* 1995, 93, 17011709). Certain angiographic data have revealed that a regular plaque profile is a fairly specific, though insensitive, indicator of thrombosis (Kaski *et al., Circulation* 1995, 92, 2058-2065). Such plaques are likely to progress to complete occlusion, while others are equally likely to progress, but less often reach the point of complete occlusion (Aldeman *et al., J. Am. Coll. Cardiol.* 1993, 22, 1141-1154). Those that do abruptly progress to occlusion actually account for most myocardial infarctions (Ambrose *et al., J. Am. Coll. Cardiol.* 1988, 12, 56-62; Little *et al., Circulation* 1988, 78, 1157-1166). One of the major limitations of angiography is that diffuse atherosclerotic disease may narrow the entire lumen of the artery, and as a result, angiography underestimates the degree of stenosis.

[0012] The size of the plaque occlusion is not necessarily determinative. Studies show that most occlusive thrombi are found over a ruptured or ulcerated plaque that is estimated to have produced a stenosis of less than 500 of the vessel diameter. Such stenoses are not likely to cause angina or result in a positive treadmill test. In fact, most patients who die of myocardial infarction do not have three-vessel disease or severe left ventricular dysfunction (Farb *et al., Circulation* 1995, 93, 1701-1709).

[0013] Angioscopy is another technique for the visualization of artery walls, rather than the lumen, and for the characterization of atherosclerotic disease. The angioscopy technique reveals the plaque and surface features not seen by angiography. In addition, it allows the observation of the color (red, white or yellow) of the material in the artery, and is therefore highly sensitive for the detection of thrombus. However, it views only the lesion surface and is not representative of the internal heterogeneity of the plaque. As a routine clinical tool, it may not be practical due to the thickness of the catheter and the invasiveness of this technique. U.S. Patent No. 5,217,456 and U.S. Patent No. 5,275,594 disclose the use of light that induces fluorescence in tissues, and of laser energy that stimulates fluorescence in non-calcified tissues. These types of devices differentiate healthy tissue from atherosclerotic plaque, but are

not clinically useful for differentiating vulnerable plaque from less dangerous, stable plaque.

[0014] High-resolution, real-time B-mode ultrasonography with Doppler flow imaging (Duplex scanning) has merged as one of the best modalities for visualization of carotid arteries (Patel *et al., Stroke* 1995, 26, 1753-1758). Measurements of wall thickness and quantitative analysis of plaque mass and area can be determined. The echogenicity of the plaque reflects plaque characteristics; echoluscent heterogeneous plaque is associated with both intraplaque hemorrhage and lipids, whereas echodense homogeneous plaque is mostly a fibrous plaque. In addition, the configuration of the plaque (mural versus nodular) can identify active (mural) lesions that are more prone to proliferation and thromboembolism (Weinberger *et al., J. Am. Med. Assoc.* 1995, 12, 1515-1521). Because the technique is not invasive, it can be used to evaluate the efficacy of drug treatment and to study the natural history of atheroscolerosis (longitudinal studies) by follow-up of individuals at increased risk of atherosclerosis. In coronary and peripheral arteries of low extremities, however, Duplex scanning is clinically not as useful as the traditional angiography.

[0015] Atherosclerotic calcification is an organized and regulated process and is found more frequently in advanced lesions, although it may occur in small amount in early lesions (Erbel *et al., Eur. Heart* J. 2000, 21, 720-732; Wexler *et al., Circulation* 1996, 94, 11751192). There is a strong association between coronary calcium and obstructive coronary artery disease, and is clearly shown that the amount of coronary calcium was a useful predictor of the extent of coronary artery disease (Agatson *et al., J. Am. Coll. Cardiol.* 1990, 15, 827-832; Schmermund *et al., Am. J. Cardiol. 2000, 86,* 127-132; Budoff *et al., Am. J. Cardiol. 2000, 86,* 8-11). MRI, fluoroscopy, electron beam CT (EBCT), and helical CT can identify calcific deposits in blood vessels. However, only EBCT can quantitate the amount or volume of calcium (Wexler *et al., Circulation* 1996, 94, 1175-1192). In addition, the EBCT images of the myocardium can be obtained in 0.1 sec. Because of the rapid image acquisition time, motion artifacts are eliminated (Brundage *et al.* In: Fuster, V. (Ed.). *Syndromes of Atherosclerosis: correlations of clinical imaging and pathology.* Armonk, NY:

Futura Publishing Company, Inc., 1996, 417-427). It has been well-documented that the presence of coronary artery calcium, detected by EBCT, may be a sensitive early marker for the presence and progression of atheroclerotic lesion before the development of complicated lesions (Janowitz *et al., Am. J. Cardiol.* 1993, 72, 247-254).

[0016] A major limitation using EBCT for the characterization of calcium in the plaque is reproducibility (Becker *et al., Eur. Radiol. 2000, 10,* 629-635). In particular the reproducibility of small and very small calcium scores (<100) is lower than that for higher score values. In addition, coronary calcium screening can not reveal atherosclerotic plaque that has little or no calcification-and such soft, lipid-rich plaques are perhaps the most dangerous of all, vulnerable to rupture as a result of hemodynamic stress or inflammation (Carrington, C., *Diagnostic imaging, 2000,* (April), 48-53; Doherty *et al., Am. Heart J.* 1999, 137, 806-814).

[0017] As red blood cells and platelets gather at the site of the rupture, a blood clot forms and blocks the artery, causing a heart attack. Biologically, calcium may not be the ideal marker because a calcified lesion is presumably a stable lesion, less prone to rupture. More recent data show that coronary calcium scores do not seem to predict myocardial perfusion deficits, plaque burden, or cardiovascular events (Rumberger, J. A. *Circulation 1998, 97, 2095-2097;* Polak, J. F. *Radiology 2000, 216, 323-324).*

[0018] Magnetic resonance techniques using gradient echo methods to generate images of flowing blood as positive contrast within the lumen of vessels are similar to conventional angiography techniques (Doyle, M. and Pohost, G. In: Fuster, V. (Ed.). *Syndromes of Atherosclerosis: correlations of clinical imaging and pathology.* Armonk, NY: Futura Publishing Company, Inc., *1996, 313-332;* Grist, T. and Turski, P. A. In: Fuster, V. (Ed.). *Syndromes of Atherosclerosis: correlations of clinical imaging and pathology.* Armonk, NY: Futura Publishing Company, Inc., *1996, 333-362).* Magnetic resonance angiography (MRA) of coronary arteries is currently under development, and the resolution is within the range of 1 mm^3. MRA techniques provide images of the vessel lumen, whereas MRI studies are often performed to evaluate the effects of the disease on the tissue supplied by the vessel. Recent

developments in high-resolution (0.4 mm), fast spin-echo imaging and computer processing techniques visualize in vivo, atherosclerotic plaque activity and intimal thickening (Yuan *et al.*, *J. Magn. Reson. Imaging* 1994 *4,* 43-49).

[0019] In a recent clinical study in patients with carotid atherosclerosis, MRT was the first non-invasive imaging modality to allow the discrimination of lipid cores, fibrous caps, calcification, normal media, adventia, intraplaque hemorrhage, and acute thrombosis (Toussaint *et al.*, *Atheroscler. Thromb.* 1995, *15,* 15331542; Toussaint *et al., Circulation* 1996, *94*, 932-938). The key advantage of contrast-enhanced rapid imaging techniques is the ability to provide detailed "functional information" with high accuracy (McVein, E. R. *Magn. Reson. Imaging* 1996, *14*, 137-150; Glover, G. D. and Herfkins, R. *J. Radiology* 1998, *207,* 289-235).

[0020] In the last two decades, many radiotracers have been developed based on several molecules and cell types involved in atherosclerosis. The potential utility of these radiotracers for imaging atherosclerotic lesions has been studied in animal models, and has been recently reviewed (Vallabhajosula, S. and Fuster, *V. J. Nucl. Med.* 1997, *38,* 1788-1796; Cerqueira, M. *D. Seminars Nucl. Med.* 1999, *29,* 339-351; Narula, J. *J. Nucl. Cardiol.* 1999, *6,* 81-90; Narula, J. *Nucl. Med. Commun.* 2000, *21,* 601-608). In general, radiolabeled proteins and platelets have shown some clinical potential as imaging agents of atherosclerosis, but due to poor target/background and target/blood ratios, these agents are not ideal for imaging coronary or even carotid lesions. Radiolabeled peptides, antibody fragments and metabolic tracers like FDG appear to offer new opportunities for nuclear scintigraphic techniques in the noninvasive imaging of atherothrombosis. However, noninvasive imaging of atherosclerosis remains a challenge for nuclear techniques mainly due to their intrinsic shortcomings, such as low resolution, compared to MRI and CT.

[0021] Most of these techniques identify some of the morphological and functional parameters of atherosclerosis and provide qualitative or semiquantitative assessment of the relative risk associated with the disease. Knowledge of the composition of an atherosclerotic plaque may provide a window on the progression of the lesion, which may result in the development of specific therapeutic strategies for intervention.

However, these diagnostic procedures are either invasive or yield little information on the underlying pathophysiology such as cellular composition of the plaque, and biological characteristics of each component in the plaque at the molecular level.

[0022] As such, a non-invasive method to diagnose and monitor various cardiovascular diseases (*e.g.*, atherosclerosis, vulnerable plaque, coronary artery disease, renal disease, thrombosis, transient ischemia due to clotting, stroke, myocardial infarction, organ transplant, organ failure and hypercholesterolemia) are needed. The non-invasive method should yield information regarding the underlying pathophysiology of the plaque, such as the cellular composition of the plaque and biological characteristics of each component in the plaque at the molecular level.

[0023] The principal mechanisms involved in atherogenesis are lipid infiltration, cellular invasion and proliferation and thrombus formation. Molecular imaging of atherosclerotic lesions is expected to target one of the three major components of plaque-lipid core, macrophage infiltration or proliferating smooth muscle cells (Ross, R. *Nature* 1993, *362*, 801-809). Since predominance of any one component determines the behavior of the plaque, it is logical to assume that detection of an abundance of a given component will address the prognostic outcome of the plaque (Ross). The presence of large necrotic lipid cores contributes to the vulnerability of plaque to rupture. The intense macrophage infiltration of the plaque leads to release of cytokines and matrix metalloproteinases and thereby renders the plaque prone to rupture. The prevalence of smooth muscle cells provide stability to the plaque, but rapid proliferation is associated with rapidly progressive luminal stenosis such as in post-angioplastic restenosis. Therefore, it is possible to selectively target one of these three components for molecular imaging of atherosclerosis.

[0024] Oxidized LDL (oxLDL) is strongly implicated in the pathobiology of atherosclerosis. It is suspected that the lipid pool in atherosclerotic plaque is due to uptake of oxLDL, not native LDL. OxLDL is recognized by scavenger receptors on macrophages; uptake of large quantities of oxLDL by macrophages can give rise to foam cells which are an important component of atherosclerotic plaque. It is believed that foam cells

may give rise to the lipid core of vulnerable plaque, (Steinberg, D. *J. Biol. Chem.* 1997 *272* 29063-29066).

[0025] Several technologies have been described to target oxLDL or its receptors in order to detect or image atherosclerotic plaque. An antibody to oxLDL has been radiolabeled with 125I and shown to localize in atherosclerotic plaque (Tsimikas, S. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2000, 20:689-697). OxLDL, itself has been labelled and observed to concentrate in atherosclerotic plaque (Shaish, A. *et al.*, *Pathobiology* 2001 69:225-229; Iuliano, L. *et al.*, *Atherosclerosis* 1996 126:131-141). The SR-A macrophage scavenger receptor is known to bind oxLDL in order for the oxLDL to be internalized for destruction. This is the process by which foam cells are formed when macrophages are overwhelmed by oxLDL. Agonists to this receptor have been labeled and shown to be a useful imaging agent for atherosclerotic plaque (WO 2002/06771). None of these technologies have been shown to be entirely satisfactory for the early detection/imaging of vulnerable atherosclerotic plaque. The early detection and imaging of vulnerable plaque in asymptomatic patients remains a significant unsolved problem in medicine and therefore, the development of additional agents for the early detection and imaging of vulnerable plaque is warranted.

[0026] LOX-1 or lectin-like oxidized LDL receptor was recently identified as a receptor on endothelial cells for oxLDL; it mediates the internalization of oxLDL by endothelial cells and is distinct from macrophage scavenger receptors such as those described in WO 2002/06771, (Sawamura, T. *Nature* 1997 386:73-77). The amino acid sequence of LOX-1 is shown in Figure 3. LOX-1 also is expressed on macrophages and may play a role in oxLDL recognition/internalization on these cells (Yoshida, H. *et al.*, *Biochem. J.* 1998 334:9-13). LOX-1 is nearly undetectable in healthy human aorta samples but is found in atherosclerotic plaque, particular early lesions that are unlikely to be detectable by other means (Kataoka, H. *et al.*, *Circulation* 1999 99:3110-3117). An antibody to LOX-1 has been developed described as being useful to treat atherosclerosis by preventing binding of oxLDL to LOX-1 (WO0164862). Recent work suggests that recognition of oxLDL by LOX-1 is a critical early step in expression of adhesion receptors on endothelial cells. These receptors are believed to

be responsible for attracting monocytes to the early atherosclerotic plaque. The monocytes penetrate the endothelial, differentiate into macrophages and can end up as foam cells in the growing plaque. Finally, peptides were developed that bind to LOX-1 using phage display technology (White, S. *et al., Hypertension* 2001 37:449-455).

[0027] EP 1 046 652 A1 discloses a fusion polypeptide composed of an extracellular domain of mammalian oxidized-LDL receptor (LOX-1) and a part of IgG, whereby the fusion polypeptide may be labeled with a labeling agent. Thus, the fusion polypeptide can be used to detect, quantify, separate, and purify oxidized LDL. The fusion polypeptides can not be used to detect or quantify LOX-1.

[0028] The description herein of disadvantages and deleterious properties and/or results achieved with known products, methods, and apparatus, is in no way intended to limit the scope of the invention. Indeed, the present invention may utilize one or more known products, methods, and apparatus without suffering from the described disadvantages and deleterious properties and/or results.

SUMMARY OF THE INVENTION

[0029] There is a need to develop an imaging agent/molecule that is capable of binding LOX-1 and being imaged by external non-invasive imaging techniques. There also is a need to develop a method of making such an imaging agent/molecule, as well as a method of imaging a subject to assess the presence of a disease or lesion in a patient or the risk of the patient having the disease or lesion in the future. Diseases envisioned include: atherosclerosis, vulnerable plaque, coronary artery disease, renal disease, thrombosis, transient ischemia due to clotting, stroke, myocardial infarction, organ transplant, organ failure and hypercholesterolemia. It therefore is a feature of the invention to provide an imaging agent/molecule that is capable of binding LOX-1 *in vivo* to enable the detection of, and hence, quantitation of the expression of the LOX-1 protein.

[0030] In accordance with these and other features of various embodiments of the invention, there is provided a compound having the formula $S\text{-}(L)_n\text{-}B$, wherein S provides a

signal that can be detected *in vivo* or detected *in vitro*, L links S to B, B is an agent that binds to LOX-1, and n is either 0 or 1. It is preferred that B is an agent other than a peptide.

[0031] In accordance with another feature of an embodiment of the invention, there is provided a method of making a compound having the formula S-$(L)_n$-B, wherein S provides a signal that can be detected *in vivo* or detected *in vitro*, L links S to B, B is an agent that binds to LOX-1, and n is either 0 or 1, the method comprising synthesizing an agent B that binds to LOX-1; optionally synthesizing a ligand (L); and attaching a signal-generating component (S) to the agent B or ligand L. In the above formula, it is preferred that B is an agent other than a peptide.

[0032] In accordance with another feature of an embodiment of the invention, there is provided a composition comprising the above-described compound. Another feature of the invention is a method of detecting and quantifying LOX-1 in a mammal comprising administering to a mammal suspected of a disease or disorder caused by expression of LOX-1 the above-described composition, imaging the mammal, and detecting the presence and relative quantity of LOX-1 in the imaged area. The method also includes repeating the above procedure periodically to monitor the quantity of LOX-1, thereby monitoring the efficacy of therapies for treating diseases or disorders caused by expression of LOX-1.

[0033] These and other features of the invention will be readily apparent to those skilled in the art upon reading the detailed description that follows:

BRIEF DESCRIPTION OF THE DRAWINGS

[0034] FIG. 1 is an illustration of L and S variation in the S-L-B composition.

[0035] FIG 2. is a transmitted light image overlayed against a fluorescent confocal image of human coronary artery endothelial (HCAE) cells after being contacted with a molecule of the present invention and imaged.

[0036] FIG. 3 is the DNA sequence for human LOX-1.

[0037] FIG. 4 is a fluorescent image and FIG. 5 is a transmitted light image and of HCAE cells bound to fluorescein-labeled polyclonal antibody for LOX-1.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

[0038] Embodiments of the invention are not limited to the particular methodology, protocols, cell lines, vectors, and reagents described in the preferred embodiments, as these may vary. It also is to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of any embodiment of the present invention, which will be limited only by the appended claims.

[0039] As used throughout this disclosure, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a host cell" includes a plurality of such host cells, and a reference to "an antibody" is a reference to one or more antibodies and equivalents thereof known to those skilled in the art, and so forth.

[0040] Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are now described. All publications mentioned herein are cited for the purpose of describing and disclosing the various molecules, amino acid sequences, cell lines, vectors, and methodologies that are reported in the publications and that might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosures by virtue of prior invention.

[0041] Throughout this description, the phrase "S provides a signal that can be detected *in vivo* or detected *in vitro*" denotes an entity that can be imaged by itself or by reacting

with another substance, and that can be detected *in vivo* or *in vitro* by a detection apparatus. More specifically, the labeling entities S include enzymes, fluorescent materials, chemiluminescent materials, biotin, avidin, radioisotopes, radionuclides, X-ray imaging agents, MRI contrast agents, ultrasonography imaging elements, paramagnetic materials, and the like. Suitable imaging agents that provide a detectable signal (S) will be described in more detail below.

[0042] Any binding moiety (B) can be used so long as it is capable of binding to LOX1. The binding moiety may include, for example:

- micellular particles like LDL, oxLDL and derivatives
- polynucleotides and derivatives such as polyinosinic acid
- proteins particularly including monoclonal and polyclonal antibodies, antibody fragments, diabodies, and the like
- protein derivatives such as glycosylated proteins
- polysaccharides or derivative of polysaccharides such as carrageenan and dextran sulfate
- peptides or peptide derivatives
- low molecular weight molecules

[0043] The term "fragment" refers to a protein or polypeptide that consists of a continuous subsequence of the subject amino acid sequence and includes naturally occurring fragments such as splice variants and fragments resulting from naturally occurring *in vivo* protease activity. Such a fragment may be truncated at the amino terminus, the carboxy terminus, and/or internally (such as by natural splicing). Such fragments may be prepared with or without an amino terminal methionine.

[0044] The term "variant" refers to a protein or polypeptide in which one or more amino acid substitutions, deletions, and/or insertions are present as compared to the subject amino acid sequence and includes naturally occurring allelic variants or alternative splice variants. The term "variant" includes the replacement of one or more amino acids in a peptide sequence with a similar or homologous amino acid(s) or a dissimilar amino acid(s). There are many scales on which amino acids can be ranked as similar or

homologous. (Gunnar von Heijne, *Sequence Analysis in Molecular Biology,* p. 123-39 (Academic Press, New York, NY 1987.) Preferred variants include alanine substitutions at one or more of amino acid positions. Other preferred substitutions include conservative substitutions that have little or no effect on the overall net charge, polarity, or hydrophobicity of the protein. Conservative substitutions are set forth in Table 1 below.

TABLE 1

Conservative Amino Acid Substitutions

Basic:	arginine
	lysine
	histidine
Acidic:	glutamic acid
	aspartic acid
Uncharged Polar:	glutamine
	asparagine
	serine
	threonine
	tyrosine
Non-Polar:	phenylalanine
	tryptophan
	cysteine
	glycine
	alanine
	valine
	proline
	methionine
	leucine
	isoleucine

[0045] Table 2 sets out another scheme of amino acid substitution:

TABLE 2

Original Residue	Substitutions
Ala	gly; ser
Arg	lys
Asn	gln; his
Asp	glu
Cys	ser
Gln	asn
Glu	asp
Gly	ala; pro
His	asn; gln
Ile	leu; val
Leu	ile; val
Lys	arg; gln; glu
Met	leu; tyr; ile
Phe	met; leu; tyr
Ser	thr
Thr	ser
Trp	tyr
Tyr	trp; phe
Val	ile; leu

[0046] Other variants can consist of less conservative amino acid substitutions, such as selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. The substitutions that in general are expected to have a more significant effect on function are those in which (a) glycine and/or proline is substituted by another amino acid or is deleted or inserted; (b) a hydrophilic residue, *e.g.*, seryl or threonyl, is substituted for (or by) a hydrophobic residue, *e.g.*, leucyl, isoleucyl, phenylalanyl, valyl, or alanyl; (c) a cysteine residue is substituted for (or by) any other residue; (d) a residue having an electropositive side chain, *e.g.*, lysyl, arginyl, or histidyl, is substituted for (or by) a residue having an electronegative charge, *e.g.*, glutamyl or aspartyl; or (e) a residue having a bulky side chain, *e.g.*, phenylalanine, is substituted for (or by) one not having such a side chain, *e.g.*, glycine.

[0047] Other variants include those designed to either generate a novel glycosylation and/or phosphorylation site(s), or those designed to delete an existing glycosylation and/or phosphorylation site(s). Variants include at least one amino acid substitution at a glycosylation site, a proteolytic cleavage site and/or a cysteine residue. Variants also include peptides with additional amino acid residues before or after the subject amino acid sequence on linker peptides. For example, a cysteine residue may be added at both the amino and carboxy terminals of a subject amino acid sequence in order to allow the cyclisation of the subject amino acid sequence by the formation of a di-sulphide bond. The term "variant" also encompasses polypeptides that have the subject amino acid sequence with at least one and up to 25 or more additional amino acids flanking either the 3' or 5' end of the subject amino acid.

[0048] The term "derivative" refers to a chemically modified protein or polypeptide that has been chemically modified either by natural processes, such as processing and other post-translational modifications, but also by chemical modification techniques, as for example, by addition of one or more polyethylene glycol molecules, sugars, phosphates, and/or other such molecules, where the molecule or molecules are not naturally attached to wild-type amino acids so derivatized. Derivatives include salts. Such chemical modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature, and they are well known to those of skill in the art. It will be appreciated that the same type of modification may be present in the same or varying degree at several sites in a given protein or polypeptide.

[0049] In addition, a given protein or polypeptide may contain many types of modifications. Modifications may take place anywhere in a protein or polypeptide, including the peptide backbone, the amino acid side-chains, and the amino or carboxyl termini. Modifications include, for example, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-

carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, glycosylation, lipid attachment, sulfation, γ-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins, such as arginylation, and ubiquitination. PROTEINS--STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993) and Wold, F., *Posttranslational Protein Modifications: Perspectives and Prospects*, pgs. 1-12 in *Posttranslational Covalent Modification Of Proteins*, B. C. Johnson, Ed., Academic Press, New York (1983); Seifter *et al.*, *Meth. Enzymol.* 182:626-646 (1990) and Rattan *et al.*, *Protein Synthesis: Posttranslational Modifications and Aging*, Ann. N.Y. Acad. Sci. 663: 48-62 (1992). The term "derivatives" include chemical modifications resulting in the protein or polypeptide becoming branched or cyclic, with or without branching. Cyclic, branched and branched circular proteins or polypeptides may result from post-translational natural processes and may be made by entirely synthetic methods, as well.

[0050] The term "homologue" refers to a protein that is at least 60 percent identical in its amino acid sequence of the subject amino acid sequence, as the case may be, as determined by standard methods that are commonly used to compare the similarity in position of the amino acids of two polypeptides. The degree of similarity or identity between two proteins can be readily calculated by known methods, including but not limited to those described in COMPUTATIONAL MOLECULAR BIOLOGY, Lesk, A. M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D. W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data*, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo H. and Lipman, D., SIAM, *J. Applied Math.*, 48: 1073 (1988). Preferred methods to determine identity are designed to provide the largest match between the sequences tested. Methods to

determine identity and similarity are codified in publicly available computer programs.

[0051] Preferred computer program methods useful in determining the identity and similarity between two sequences include, but are not limited to, the GCG program package (Devereux, J., *et al.*, *Nucleic Acids Research,* 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA, Atschul, S. F. *et al.*, *J. Molec. Biol.*, 215: 403-410 (1990). The ***BLAST*** X program is publicly available from NCBI and other sources (***BLAST*** Manual, Altschul, S., *et al.*, NCBI NLM NIH Bethesda, Md. 20894; Altschul, S., *et al., J. Mol. Biol.*, 215: 403-410 (1990). By way of example, using a computer algorithm such as GAP (Genetic Computer Group, University of Wisconsin, Madison, Wis.), the two proteins or polypeptides for which the percent sequence identity is to be determined are aligned for optimal matching of their respective amino acids (the "matched span", as determined by the algorithm).

[0052] A gap opening penalty (which is calculated as 3 x (times) the average diagonal; the "average diagonal" is the average of the diagonal of the comparison matrix being used; the "diagonal" is the score or number assigned to each perfect amino acid match by the particular comparison matrix) and a gap extension penalty (which is usually 1/10 times the gap opening penalty), as well as a comparison matrix such as PAM 250 or BLOSUM 62 are used in conjunction with the algorithm. A standard comparison matrix (*see* Dayhoff *et al.* in: *Atlas of Protein Sequence and Structure*, vol. 5, supp.3 [1978] for the PAM250 comparison matrix; see Henikoff *et al.*, *Proc. Natl. Acad. Sci USA*, 89:10915-10919 [1992] for the BLOSUM 62 comparison matrix) also may be used by the algorithm. The percent identity then is calculated by the algorithm. Homologues will typically have one or more amino acid substitutions, deletions, and/or insertions as compared with the comparison subject amino acid, as the case may be.

[0053] The term “fusion protein” or “fusion polypeptide” refers to a protein where one or more of the subject amino acid sequences are recombinantly fused or chemically conjugated (including covalently and non-covalently) to a protein such as (but not limited to) an antibody or antibody fragment like an F_{ab} fragment or short chain Fv.

The term “fusion protein” or “fusion polypeptide” also refers to multimers (i.e. dimers, trimers, tetramers and higher multimers) of peptides. Such multimers comprise homomeric multimers comprising one subject peptide, heteromeric multimers comprising more than one subject peptide, and heteromeric multimers comprising at least one subject peptide and at least one other protein. Such multimers may be the result of hydrophobic, hyrdrophilic, ionic and/or covalent associations, bonds or links, may be formed by cross-links using linker molecules or may be linked indirectly by, for example, liposome formation. Various fusion proteins known to bind to the human LOX-1 receptor are disclosed in EP 1046652A1, the disclosure of which is incorporated by reference herein in its entirety.

[0054] The term “peptide mimetic” or “mimetic” refers to biologically active compounds that mimic the biological activity of a peptide or a protein but are no longer peptidic in chemical nature, that is, they no longer contain any peptide bonds (that is, amide bonds between amino acids). Here, the term peptide mimetic is used in a broader sense to include molecules that are no longer completely peptidic in nature, such as pseudo-peptides, semi-peptides and peptoids. Examples of peptide mimetics in this broader sense (where part of a peptide is replaced by a structure lacking peptide bonds) are described below. Whether completely or partially non-peptide, peptide mimetics according to this invention provide a spatial arrangement of reactive chemical moieties that closely resemble the three-dimensional arrangement of active groups in the subject peptide on which the peptide mimetic is based. As a result of this similar active-site geometry, the peptide mimetic has effects on biological systems that are similar to the biological activity of the subject peptide.

[0055] The peptide mimetics of this invention are preferably substantially similar in both three-dimensional shape and biological activity to the subject peptides described herein. Examples of methods of structurally modifying a peptide known in the art to create a peptide mimetic include the inversion of backbone chiral centers leading to D-amino acid residue structures that may, particularly at the N-terminus, lead to enhanced stability for proteolytical degradation without adversely affecting activity. An example is given in the paper “Tritriated D-ala^1 -Peptide T Binding”, Smith C. S. *et al.*, *Drug Development Res.*, 15, pp. 371-379 (1988). A second method is altering

cyclic structure for stability, such as N to C interchain imides and lactames (Ede *et al.* in Smith and Rivier (Eds.) "Peptides: Chemistry and Biology", Escom, Leiden (1991), pp. 268-270). An example of this is given in conformationally restricted thymopentin-like compounds, such as those disclosed in U.S. Pat. No. 4,457,489 (1985), Goldstein, G. *et al.*, the disclosure of which is incorporated by reference herein in its entirety. A third method is to substitute peptide bonds in the subject peptide by pseudopeptide bonds that confer resistance to proteolysis.

[0056] A number of pseudopeptide bonds have been described that in general do not affect peptide structure and biological activity. One example of this approach is to substitute retro-inverso pseudopeptide bonds ("Biologically active retroinverso analogues of thymopentin", Sisto A. *et al* in Rivier, J. E. and Marshall, G. R. (eds) "Peptides, Chemistry, Structure and Biology", *Escom, Leiden* (1990), pp. 722-773) and Dalpozzo, *et al.* (1993), *Int. J. Peptide Protein Res.*, 41:561-566, incorporated herein by reference). According to this modification, the amino acid sequences of the peptides may be identical to the sequences of the subject amino acid sequence, except that one or more of the peptide bonds are replaced by a retro-inverso pseudopeptide bond. Preferably the most N-terminal peptide bond is substituted, since such a substitution will confer resistance to proteolysis by exopeptidases acting on the N-terminus. Further modifications also can be made by replacing chemical groups of the amino acids with other chemical groups of similar structure. Another suitable pseudopeptide bond that is known to enhance stability to enzymatic cleavage with no or little loss of biological activity is the reduced isostere pseudopeptide bond (Couder, *et al.* (1993), *Int. J. Peptide Protein Res.*, 41:181-184, incorporated herein by reference in its entirety).

[0057] Thus, the amino acid sequences of these peptides may be identical to the sequences of the subject amino acid sequence, except that one or more of the peptide bonds are replaced by an isostere pseudopeptide bond. Preferably the most N-terminal peptide bond is substituted, since such a substitution would confer resistance to proteolysis by exopeptidases acting on the N-terminus. The synthesis of peptides with one or more reduced isostere pseudopeptide bonds is known in the art (Couder, *et al.* (1993), cited

above). Other examples include the introduction of ketomethylene or methylsulfide bonds to replace peptide bonds.

[0058] Peptoid derivatives represent another class of peptide mimetics that retain the important structural determinants for biological activity, yet eliminate the peptide bonds, thereby conferring resistance to proteolysis (Simon, *et al.*, 1992, *Proc. Natl. Acad. Sci. USA*, 89:9367-9371, incorporated herein by reference in its entirety). Peptoids are oligomers of N-substituted glycines. A number of N-alkyl groups have been described, each corresponding to the side chain of a natural amino acid (Simon, *et al.* (1992), cited above). Some or all of the amino acids of the subject molecules may be replaced with the N-substituted glycine corresponding to the replaced amino acid.

[0059] The term "peptide mimetic" or "mimetic" also includes reverse-D peptides and enantiomers. The term "reverse-D peptide" refers to a biologically active protein or peptide consisting of D-amino acids arranged in a reverse order as compared to the L-amino acid sequence of the subject peptide. The term "enantiomer" refers to a biologically active protein or peptide where one or more the L-amino acid residues in the amino acid sequence of a subject peptide is replaced with the corresponding D-amino acid residue(s).

[0060] A "composition" as used herein, refers broadly to any composition containing a described molecule, peptide, or amino acid sequence. The composition may comprise a dry formulation, an aqueous solution, or a sterile composition. Compositions comprising the molecules described herein may be stored in freeze-dried form and may be associated with a stabilizing agent such as a carbohydrate. In use, the composition may be deployed in an aqueous solution containing salts, *e.g.*, NaCl, detergents, *e.g.*,sodium dodecyl sulfate (SDS), and other components, *e.g.*, Denhardt's solution, dry milk, salmon sperm DNA, etc.

[0061] An embodiment of the present invention relates to molecules useful in detecting or imaging atherosclerotic tissue by binding to LOX-1. These molecules preferably have the following characteristics:

- contain a moiety that binds to LOX-1 in the presence of human fluids with adequate specificity such that atherosclerotic tissue may be differentiated from healthy tissue; and
- contain a signal moiety that can be detected.

[0062] LOX-1 or lectin-like oxidized LDL receptor was recently identified as a receptor on endothelial cells for oxLDL; it mediates the internalization of oxLDL by endothelial cells and is distinct from macrophage scavenger receptors such as those described in WO 2002/06771, (Sawamura, T. *Nature* 1997 386:73-77). LOX-1 also is expressed on macrophages and may play a role in oxLDL recognition/internalization on these cells (Yoshida, H. *et al., Biochem. J.* 1998 334:9-13). LOX-1 is nearly undetectable in healthy human aorta samples but is found in atherosclerotic plaque, particular early lesions that are unlikely to be detectable by other means (Kataoka, H. *et al., Circulation* 1999 99:3110-3117). An antibody to LOX-1 has been described as being useful to treat atherosclerosis by preventing binding of oxLDL to LOX-1 (WO0164862). Recent work suggests that recognition of oxLDL by LOX-1 is a critical early step in expression of adhesion receptors on endothelial cells. These receptors are believed to be responsible for attracting monocytes to the early atherosclerotic plaque. The monocytes penetrate the endothelial, differentiate into macrophages and can end up as foam cells in the growing plaque. Finally, peptides were developed that bind to LOX-1 using phage display technology (White, S. *et al., Hypertension* 2001 37:449-455).

[0063] Various species of LOX-1 have been isolated and sequenced revealing relatively significant dissimilarity interspecies (Chen, M., *et al., J. Biochem.*, 355:289-95 (2001). U.S. Patent Nos. 5,962,260 and 6,197,937, the disclosures of which are incorporated by reference herein in their entirety, disclose the amino acid sequences of human and bovine LOX-1. Using the techniques disclosed in these documents, and the guidelines provided herein, those skilled in the art are capable of isolating LOX-1 from any species, and creating molecules such as antibodies that bind to the human LOX-1.

[0064] Thus, in the above molecule, the moiety that binds to LOX-1 can be synthesized using known techniques, given the known amino acid sequence of the LOX-1 polypeptide. Moieties that bind only specific portions of LOX-1 also can be synthesized given the known and/or expected antigenic determinant or epitope binding site. Unlike known moieties that bind to LOX-1, the inventive moieties are designed to bind to LOX-1 in the presence of human fluids (*in vivo* or *in vitro*) with sufficient specificity such that tissue in which LOX-1 has been overexpressed (*e.g.*, atherosclerotic tissue) may be differentiated from healthy tissue. The inventive molecules also are bound, again in the presence of human fluids, to a signal moiety with sufficient specificity to enable detection using imaging techniques.

[0065] Moieties that bind to LOX-1 include, for example antibodies to LOX-1, such as those described in WO 0164862, and U.S. Patent No. 6,197,937, macromolecules other than antibodies such as poly I and carrageenan (*Arterioscler Thromb Vasc Biol.* **1998** *18*:1541-1547.), peptides such as those described in White, S. *et al. Hypertension* **2001** *37* 449-455, peptide mimetics and organic molecules that satisfy the criteria above. It is preferred in an embodiment of the present invention, however, that the moieties that bind to LOX-1 do not include peptides. Preferably, the agents that bind LOX-1 are selected from antibodies, proteins, glycosylated proteins, biomolecules, polysaccharides, peptidomimetics, low molecular weight organic compounds, and mixtures, derivative, fragments, homologues, and variants thereof. A suitable protein includes Heat Shock Protein 70 (Hsp70). Delneste, Y., *et al.*, "*Involvement of LOX-1 in dendritic cell-mediated antigen cross presentation,*" *Immunity*, **17(3)**, pp 353-62 (2002).

[0066] The methods disclosed in the above documents can be used to generate a plurality of agents capable of binding LOX-1. These agents then can be screened as described herein by reacting them with a plurality of signal moieties and optional linking ligands, and tested to assess their efficacy in binding both the LOX-1 polypeptide and the signal moieties. While peptides are not preferred for use in certain embodiments of the invention, if a peptide were used, it is preferred that the peptide have one or more peptidic sequences selected from the group consisting of LSIPPKA, FQTPPQL,

LTPATAI, and mixtures, fragments, fusion peptides, derivatives, variants, and homologues thereof.

[0067] A number of methods can be used to screen and evaluate the binding affinity of different ligands. One method for example includes fluorescent based *in vitro* experiments. Cell-based assays can simultaneously yield information on the amount of signal generating entity necessary for detection, and therefore required for conjugation to ligands.

[0068] In the case of the peptidic ligands, a fluorescent dye preferably is attached to the N-terminus of the peptide via a flexible linker, such as the amino acid sequence KKGG (K=Lysine, G=Glycine). In the event that the N-terminus is linked to a signaling moiety with no further functional ends for dye attachment, the dye also can be attached via the side-chain amine of a K residue incorporated into the sequence (e.g. in the linker).

[0069] Regardless of the type of screening assay used, (e.g., a generic *in vitro* model), it first is assumed that the amount of LOX-1 on the surface of a substrate is known, whether it be cells or some other substrate. In a multi-well transparent plate, LOX-1 is present (either as pure LOX-1 protein or expressed on cells) uniformly across the wells. Labeled ligands then can be added and incubated for an optimized amount of time in the different wells. The wells then are washed thoroughly with a buffer, such as Phosphate buffered saline (PBS), and the plate then imaged while shining a laser to excite and initiate fluorescence of the dye attached to the ligands. The fluorecent intensity from each well, and thus the degree of different ligand binding, can be quantified. To obtain the absolute number of ligand bound, the signal preferably is further calibrated by obtaining the fluorescent intensity of a known quantity of dye-conjugated ligand under similar conditions of the binding assay. If the number of LOX-1 molecule is known, and the amount of bound ligands determined, dissociation constants to evaluate ligand-binding affinity can be calculated. Different ligands can thus be screened quantitatively for their binding affinity. The number of bound

ligands per cells also is capable of providing information on parameters required to obtain a detectable signal from a signal-generating entity conjugated to the ligands.

[0070] Images in the assay may be acquired using a laser confocal microscope or an Imager. For example, images of peptides bound to cells can be obtained using a laser confocal microscope as follows: HCAE cells can be grown on high quality borosilicate 8-chambered glass slides (Electron Microscopy Sciences, Fort Washington, PA). Then, about 10 μL of 1mg/ml of an aqueous solution of a labeled peptide can be added to the cells and incubated for 1 hour. Subsequently, the cells preferably are washed with HBSS buffer three times. The cells then can be fixed with 4% formaldehyde solution over 10 minutes. After a final wash with buffer, the slide is imaged. Images preferably are acquired using an OLYMPUS laser scanning confocal microscope, model Fluoview 300, using Ar-ion laser (selecting 488 nm line) and a 510-nm long-pass filter. Images can be acquired using two channels: reflected light and fluorescent mode channel, or an overlay of both channels.

[0071] For higher throughput screening the method described above can be extended: a 96-well plate may replace the 8-well slides and a Biorad Imager, model FX Proplus, replace the confocal microscope. For example, images of fluorescein-labeled polyclonal antibody bound to cells can be obtained using an Imager, whereby HCAE cells can be laid on and grown in wells on a standard commercial 96-well plate (Becton-Dickenson, Franklin Lakes, NJ). Then, about 10 μL of 1mg/ml labeled antibody aqueous solution can be added to the cells and incubated for 1 hour. Subsequently, the cells preferably are washed with PBS buffer three times. After a final wash with buffer, the slide can be imaged using the Biorad imager selecting "Fluorescein" as the fluorophore..

[0072] Any signal moiety can be used so long as it is capable of binding the binding moiety and generating a detectable signal. Suitable signal moieties include a luminescent dye, a radionuclide, a near infrared dye, a magnetically active isotope, a superparamagnetic particle, a metal ion having a Z value of greater than 50, an encapsulated species, and a combination thereof. The signal moiety may include, for example:

- dyes, fluorescent dye, chemiluminescent dyes for optical imaging, histology;
- molecules containing high-Z elements, such as iodine, for X-ray imaging, computed tomography (CT);
- gas-filled microbubbles, fluorocarbon filled micelles for ultrasonography (US);
- paramagnetic ions, such as chelated Gd^{+++}, or superparamagnetic particles such as superparamagnetic iron oxide nanoparticles (SPIO) for magnetic resonance imaging (MRI); or
- radionuclides such as 99mTc for single photon emission computed tomography (SPECT) or ^{18}F for positron emission tomography (PET).

[0073] Particularly preferred signal moieties include fluorescein, ^{11}C, ^{18}F, ^{52}Fe ,^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{86}Y, ^{89}Zr, ^{94m}Tc, ^{94}Tc, ^{99m}Tc, ^{111}In, ^{123}I, ^{124}I, ^{125}I, ^{131}I, $^{154-158}Gd$ and ^{175}Lu, superparamagnetic iron oxide nanoparticles, heavy metal ions, gas-filled microbubbles, optical dyes, porphyrins, texaphyrins, highly iodinated organic compounds chelates thereof, polymers containing at least one of the aforementioned components, endoedral fullerenes containing at least one of the aformentioned, and mixtures thereof. Even more preferably, the signal moieties are ^{18}F for PET, superparamgnetic iron oxide nanoparticles (SPIO) for MRI, chelated Gd, I, and Y. Most preferably, the signal moiety is ^{18}F for PET.

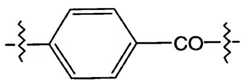
[0074] ^{18}F-Fluoride can be obtained from cyclotrons after bombardment of ^{18}O-enriched water with protons. Typically, the enriched water containing ^{18}F-fluoride is treated with a base having a counter-ion that is any alkali metal cation (M^+), such as potassium or another monovalent ion as well as a chelate for M^+, such as Kryptofix 222. The water can be evaporated off to produce a residue of chelate M-^{18}F, which can be taken up in an organic solvent for further use. The purpose of the chelate is to solubilize the M-^{18}F in the organic solvent and confer nucleophilicity to the ^{18}F-fluoride. Instead of a chelate and M^+, a quaternary ammonium salt, phosphonium salt or guandinium may be used to solubilize the ^{18}F-fluoride in the organic solvent and confer nucleophilic reactivity to the ^{18}F-fluoride. Potassium is generally used as a counter-ion

[0075] Because fluoride is the most electronegative element, it has a tendency to become hydrated and lose its nucleophilic character. To minimize this, the labeling reaction preferably is performed under anhydrous conditions. For example, fluoride (as potassium fluoride or as a complex with any of the other counter-ions discussed above) can be placed in organic solvents, such as acetonitrile or THF. With the assistance of agents that bind to the counter-ion, such as Kryptofix 2.2.2(4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]-hexacosane), the fluoride ion is very nucleophilic in these solvents. The remaining portion of the chelate molecule of the invention then can be added to the solvent and the chelate thereby labeled with the ^{18}F. Using the guidelines provided herein, those skilled in the art are capable of labeling the ligands of the present invention with ^{18}F. Alternatively, labeling may be accomplished through the use of ^{18}F-F_2 or electrophilic fluorinating agents derived from ^{18}F-F_2.

[0076] Other suitable signaling moieties include magnetically active isotopes, such a paramagnetic ions, isotopes of gadolinium, and polymers containing such compounds. Nanoparticles of iron oxides or elemental iron also can be used as superparamagnetic signaling agents. Components having a Z value greater than about 50, such as iodine and bismuth also can be used. Suitable signal moieties further include encapsulated species such as micelles, liposomes, polysomes, and gas-filled microbubles.

[0077] L is simply any moiety, which connects the signal moiety S to the binding moiety B. In the case of ^{18}F or ^{11}C a linker may not be necessary; the radioisotope can be directly attached to B via a covalent bond. In many cases it is preferred to include L in order to attach S to B. That is, n in the equation for the molecule of the invention is 1. Preferred linking agents include polypeptides, proteins, and small organic moieties. For example, lysine-glycine analogs, derivatives and variants can be used, conventional chelators such as cyclohexyl alanine, DTPA, 1,4,7-triaza-cyclononane-N,N',N"-triacetic acid (NOTA), p-bromoacetamido-benyl-tetraethylaminetetraacetic acid (TETA), 1,4,7,10-tetraazacyclododecanetetraacetic acid (DOTA), and combinations thereof. A preferred linking agent could be a lysine-glycine derivative such as KKGG

[0078] Organic moieties having a valence of at least 2 are useful as L in the above formula, including small organic moieties such as benzoate or propionate (Figure 1). The organic radical may be covalently bound to both S and B, or it may be ionically bound to S, B, or both S and B. The organic moiety suitable for use as the linking agent typically has from about 1 to about 10,000 carbon atoms, and may include, an organic radical selected from the group consisting of alkylene, arylene, cycloakylene, aminoaklylene, aminoarylene, aminocycloalkylene, thioalkylene, thioarylene, thiocycloalkylene, oxyalkylene, oxyarylene, oxycycloalkylene, acylalkylene, acylarylene, acylcycloalkylene units, and combinations thereof. A particularly preferred acylarylene unit is a 4-acylphenylene group having the structure below:



[0079] Other suitable linking agents including metal chelating agents, such as one or more of DTPA, 1,4,7-triaza-cyclononane-N,N',N''-triacetic acid (NOTA), p-bromoacetamido-benyl-tetraethylaminetetraacetic acid (TETA), 1,4,7,10-tetraazacyclododecanetetraacetic acid (DOTA), EDTA, and CHXa. It is preferred that the metal chelating agents be capable of binding to at least one metal selected from cations of ^{52}Fe ,^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{86}Y, ^{89}Zr, ^{94m}Tc, ^{94}Tc, ^{99m}Tc, ^{111}In, $^{154\text{-}158}$Gd, and ^{175}Lu.

[0080] As appreciated by those skilled in the art, various linking agents are used with certain signal moieties. For example, signal generating moieties, such as 64Cu, typically require a linking ligand, whereas ^{18}F does not. In addition, labeled prosthetic groups such as ^{18}F-fluoropropionate or ^{18}F-fluorobenzoate (Figure 1) can be used such that, once prepared, they can be conjugated to the peptide via active ester conjugation. Those skilled in the art are capable of synthesizing a suitable linking agent, if needed, together with a suitable signaling moiety, using the guidelines and synthesis techniques provided herein.

[0081] The labeled ligands of embodiments of the present invention can be used as a diagnostic to assist in imaging a targeted tissue that is suspected of overexpressing LOX-1. The method of diagnosis therefore includes first administering to a subject a composition containing the labeled ligand of the invention. The method also optionally includes administering a clearing agent to assist in clearing any unbound antibody and fragments thereof from circulation. Depending on the particular label that has been labeled to the ligand, the appropriate imaging technique is employed to image the targeted tissue. For example, when ^{18}F is used as the labeling agent PET imaging is conducted.

[0082] The imaging method can be used as a diagnostic to detect the presence of LOX-1 in human tissue. In addition, the imaging method can be repeated over a number of days to provide a quantitative assessment of the degree of growth or expression of the LOX-1 polypeptide.

[0083] Embodiments of the invention also encompass a composition comprising the labeled ligands, as well as a kit for imaging a targeted tissue. The kit preferably comprises a composition comprising the labeled ligand of the invention, or optionally, comprises two compositions; one containing an ^{18}F precursor, and the other containing the remaining portion of the labeled ligand. These two compositions can be mixed just prior to administration to the subject, thereby preserving the life of the ^{18}F radionuclide.

[0084] Methods of synthesizing peptidic ligand linkers (L) that are useful in labeling moieties (B) that recognize LOX-1, as well as methods of directly labeling binding agents that bind LOX-1 are described hereinafter.

[0085] Peptides can be synthesized using standard solid phase techniques with N^{α}-Fmoc-protected amino acids (Sheppard, R.C., *Peptides*, North-Holland Publishing Company, Amsterdam (1973)) using 2,4-dimethoxybenzhydrylamine resin (Rink Amide AM) on a 25 μmole scale (Fmoc = Fluorenylmethoxycarbonyl). The peptides can be synthesized using a Rainin/Protein Technology Symphony solid phase peptide synthesizer (Woburn, MA). Prior to any reaction chemistry, the resin preferably is

swelled for one hour in methylene chloride, and subsequently exchanged out with DMF (dimethylformamide) over half-hour or more.

[0086] Each coupling reaction to synthesize the peptide can be carried out at room temperature in DMF with five equivalents of amino acid. Reaction times usually are from about 20 minutes to about 3 hours, more preferably about 45 minutes, 1 hour for residues that were expected to be difficult to couple (for example, coupling Isoleucine, I, to proline, P, in the IPP sequence). The preferred coupling reagent used is HBTU (*O*-Benzotriazolyl-1-yl-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate), with NMM (N-methylmorpholine) as the base. During each coupling step, the coupling agent preferably is delivered at a scale of five equivalents relative to the estimated resin capacity, and reaction carried out in 2.5 ml of 0.4 M NMM solution in DMF.

[0087] It is preferred that the coupling reactions do not perturb the side-chains of the amino acids because the acids typically were protected with acid labile groups if reactive groups were present. For example, tyrosine, threonine and serine side chains can be protected as the corresponding *tert*-butyl ethers. Glutamic acid side chains can be protected as the corresponding *tert*-butyl ester. Lysine and ornithine side chains can be Boc protected. Glutamine side chains also may be protected as the γ-triphenylmethyl derivative, and the arginine side chain protected as the 2,2,5,7,8-Pentamethyl-chromane-6-sulfonyl derivative.

[0088] Following each coupling reaction, the N-terminal Fmoc-protected amine preferably is deprotected by applying 20% piperidine in DMF twice at room temperature for approximately 15 minutes. After the addition of the last residue the resin, still on the peptide synthesizer, preferably is rinsed thoroughly with DMF and methylene chloride. The signaling moiety (S) can be attached to the peptide for ultimate attachment to the binding moiety (B), or directly to B.

[0089] For example, to couple the fluorescein dye, 5(6)-carboxyfluorescein, to the N-terminus of a synthesized peptide, the dye, HBTU and NMM preferably are added to the resin in the same manner as the amino acids described above. After the reaction,

the resin preferably is thoroughly washed with DMF and methylene chloride and dried under a stream of nitrogen. A mixture containing 1 mL TFA, 2.5% TSP (triisopropylsilane) and 2.5% water can be used to cleave the peptides from the resin. The resin and mixture preferably are stirred at room temperature for approximately 3 to 4 hours. The resin beads then can be filtered off using glass wool, followed by rinsing with 2-3 ml of TFA. The peptide then preferably is precipitated with ice-cold ether (40 mL) and centrifuged (e.g., at 3000-4000 rpm) until the precipitate formed a pellet at the bottom of the centrifuge tube. The ether can be decanted, and the pellet resuspended in cold ether (40 mL) and centrifuged again — the process can be repeated two to three times. During the final wash 10 ml of Millipore water preferably is added to 30 ml of cold ether, and the mixture was centrifuged again. The ether then can be decanted, the aqueous layer containing the crude peptide then can be transferred to a round bottom flask for lyophilization. Crude yields for peptide synthesis were usually approximately 90%. No unlabeled peptide was typically observed.

[0090] Peptides preferably are purified by reverse phase semipreparative or preparative HPLC with a C4-silica column (Vydac, Hesperia, CA). The peptide chromatograms can be monitored at 220 nm, which corresponds to the absorption of the amide chromophore. Monitoring at 495 nm also can be observed to ensure the presence of the fluorescein dye on the peptide. It is preferred to use a solvent system including CH_3CN/TFA (acetonitrile/Trifluoroacetic acid; 100:0.01) and H_2O/TFA (water/Trifluoroacetic acid; 100:0.01) eluents at flow rates of 3 ml/min and 10 ml/min for semipreparative and preparative, respectively. Dissolved crude peptides in Millipore water can be injected at a scale of 1.5 mg and 5-10 mg peptide for semipreparative or preparative, respectively. The chromatogram shape was analyzed to ensure good resolution and peak shape. Gradient conditions for all peptides were typically 5 to 50% of CH_3CN/TFA (100:0.01) in 30 minutes. Purified peptide identity was confirmed by matrix-assisted laser desorption time-of-flight mass spectroscopy.

[0091] A polyclonal antibody, for example, that recognizes LOX-1 can be labeled using active ester chemistry in conjunction with the description herein as follows. An

aliquot containing 250 μg (166 μL) of the intact antibody (Immunoglobulin G, IgG) preferably is transferred to a 1.5 mL Eppendorf tube and maintained at 0°C. The solution then can be treated with $NaHCO_3$ (1M, 20 μL) and gently inverted. In a separate tube, a solution of an active ester compound preferably is prepared in either PBS or DMF using standard peptide synthesis techniques, such as those described previously. Specifically, a carboxylate containing labeling moeity can be activated using N-hydroxysuccimide, a water-soluble carbodiimide such as EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide), and a base such as $NaHCO_3$ or N-methylmorpholine (NMM). The antibody solution then preferably is treated with 5, 20 or 50 molar equivalents of the active ester solution.

[0092] Concentrations of organic solvents used preferably are minimized, generally below 15% by volume. The reaction vessel then can be permitted to warm to about room temperature over a 1 hour period and then gently inverted every 15 minutes to assure mixing. During this time a PD-10 column (Amersham Biosciences, Piscataway, NJ) preferably is equilibrated with 25 ml of PBS and eluted until the sorbent bed is exposed. The entire reaction mixture then can be transferred to the sorbent bed and eluted with PBS. The fast moving component contains the protein and appears at an approximate eluted volume of 3 mL. The resulting labeled antibody sample may be evaluated using Dot Blot techniques against the LOX-1 antigen with observation of either the fluorescent label or radioactivity depending on the label chosen. The results of this experiment confirm that antibody immunoreactivity is not compromised and that adequate incorporation of label had been accomplished. Further characterization by ITLC, PAGE gel analysis and whole cell binding of the labeled antibody can be performed as desired.

[0093] Preferred embodiments of the invention now will be explained with reference to the following non-limiting examples.

EXAMPLES

Example 1

[0094] A peptide was conjugated with fluorescein (Fl-KKGG-FQTPPQL) and was shown to bind to human endothelial coronary artery cells (HCAECs) which are known in the

literature to express LOX-1. An image of HCAECs grown in glass slides treated with this peptide obtained using a fluorescent confocal microsope is shown in Figure 2; the fluorescent image (shows fluorescently tagged peptide as bright green) is overlaid with the transmitted light image (shows outline of cells). The example reveals that the peptide above was localized on the cells. The experimental conditions for imaging the peptide-labeled HCAECs are described previously.

Example 2

[0095] A solution of polyclonal antibody (IgG) was produced by Invitrogen Corporation, (Carlsbad, CA) against the sequence Arg-Gly-Ala-Val-Tyr-Ala-Glu-Asn-Cys-Ile at a concentration of 1.5 mg/mL. Three aliquots containing 250 μg (166 μL) each were transferred to1.5 mL Eppendorf tubes and maintained at 0°C. The solutions were treated with $NaHCO_3$ (1M, 20 μL) and gently inverted. In a separate tube, a solution of 5-carboxyfluorescein-N-hydroxysuccinate ester in DMF (1 mg/mL) was prepared. The antibody solutions were treated with 5, 20 or 50 equivalents of the fluorescein/DMF solutions (3.95, 15.8 and 39 μL respectively). The highest concentration of DMF was 17%. The tubes were allowed to warm to room temperature over 1 hour and gently inverted every 15 minutes to assure mixing. During this time PD-10 columns were equilibrated with PBS and eluted until the sorbent bed was exposed.

[0096] The entire reaction mixtures were transferred to the columns and eluted with PBS. The fast moving yellow band was clearly visible and was collected in glass scintillation tubes (approximate eluted volume 3 mL). The purified labeled antibody samples were then evaluated using a Dot Blot technique against the LOX-1 antigen (Arg-Gly-Ala-Val-Tyr-Ala-Glu-Asn-Cys-Ile).

[0097] An image of HCAECs treated with this fluorescein-labeled polyclonal antibody is shown in Figures 4 and 5. The image was obtained using a laser confocal microscope as follows: HCAE cells were laid on and grown in wells on a standard commercial 96-well plate (Becton-Dickenson, Franklin Lakes, NJ). Then, about 10 μL of 1mg/ml labeled antibody aqueous solution was added to the cells and incubated for 1 hour.

Subsequently, the cells were washed with PBS buffer three times. After a final wash with buffer, the cells were imaged. Images were acquired using an OLYMPUS laser scanning confocal microscope, model Fluoview 300, using Ar-ion laser (selecting 488 nm line) and a 510-nm long-pass filter. Images were acquired using two channels: reflected light and fluorescent mode channel. Figure 4 is the fluorescent image (shows fluorescently tagged antibody as bright green) , while Figure 5 is the transmitted light image (shows the outline of cells). This example reveals that the antibody above was localized on the cells.

[0098] The results of this experiment confirmed that antibody immunoreactivity was not compromised in any of the samples and that adequate incorporation of dye had been accomplished in all cases. Further characterization by PAGE gel analysis confirmed protein integrity and that only the 150 kDa band contained the fluorescent label. Whole cell binding of the labeled antibody was then observed using the previously described binding protocol with HCAE cells.

[0099] The invention has been described with reference to specific embodiments and examples. Those skilled in the art appreciate that various modifications may be made to the invention without departing from the spirit and scope thereof.